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# Triterpenoid alkaloid derivatives from *Buxus rugulosa*

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**Abstract:** Four new triterpenoid alkaloid derivatives, buxrugulines A–D (**1**–**4**), together with four known ones (**5**–**8**), were isolated from the leaves and stems of *Buxus rugulosa*. The structures of compounds **1**–**4** were elucidated by NMR and MS spectroscopic analysis. All compounds were assayed for their cytotoxicities against HL-60, SMMC-7721, A549, MCF-7, and SW480 cells lines.

*Keywords: Buxus rugulosa*, triterpenoid alkaloid derivatives, buxruguline, cytotoxicity

## Introduction

*Buxus rugulosa*, belonging to the *Buxus* genus of the family Buxaceae, is a dwarf shrub growing in the rocky mountains in the northwest district of Yunnan Province. In previous phytochemical investigations of the genus *Buxus*, more than 220 triterpenoid alkaloid derivatives have been isolated[1,2]. This type of alkaloid showed interesting pharmacological activities such as anti-myocardial ischemia[3,4], antibacterial activities[5,6], and inhibition of cholinesterases[7–9]. In our previous studies from *Buxus* plants, new alkaloids with diverse structures and promising cytotoxic activities have been reported[10,11]. As part of this study, we have examined the stems and leaves of *B. rugulosa*, and consequently isolated four new triterpenoid alkaloid derivatives, buxrugulines A–D (**1**–**4**), along with four known ones, $N_{20}$-acetoxy-cyclovirobuxin D (**5**)[12], (+)-16$\alpha$-acetoxybuxabenzamidienine (**6**)[13,14], moenjodaramine (**7**)[9,15,16], irehine (**8**)[17]. Herein we report the isolation and structural elucidation of the new compounds, as well as cytotoxic activities of the isolates from *B. rugulosa*.

## Results and Discussion

A crude alkaloid fraction of *B. rugulosa* yielded eight triterpenoid alkaloid derivatives by repeated silica gel, amino silica gel, C-18 and Sephadex LH-20 chromatography.

Buxruguline A (**1**) was obtained as white powder. Its molecular formula, $C_{25}H_{37}NO$, was established on the basis of HRESIMS analysis ($[M + H]^+$, $m/z$ 368.2944). The $^1H$ NMR (Table 1) spectrum featured one N-methyl singlet at $\delta_H$ 2.47, four singlets at $\delta_H$ 0.80, 1.05, 1.17, and 1.26, corresponded to four tertiary methyl groups, and one doublet at $\delta_H$ 0.76 (6.5, H-21). The $^{13}C$ NMR spectrum exhibited 25 carbon signals containing six quaternary carbons (one carbonyl carbon at $\delta_C$ 206.8 and two olefinic carbons at 146.1 and 146.3), eight methines (four olefinic carbons at $\delta_C$ 127.6, 127.7, 128.4, and

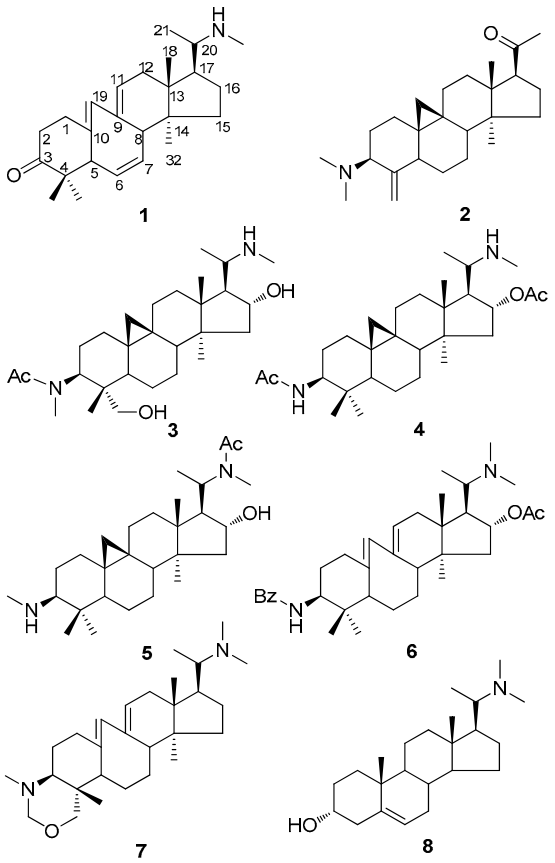


**Figure 1.** Structures of **1**–**8**.

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129.7), five methylenes, and six methyl groups. Comparison of the spectroscopic data of **1** and cyclobuxotriene[13] revealed similarities except for the absence of a methyl on the nitrogen at C-20 and the presence of a double bond at C-6/7 in **1**. This was supported by the HMBC correlations of H-5 ($\delta_H$ 2.73) with C-6 ($\delta$c 128.4) and of H-8 ($\delta_H$ 1.91) with C-7 ($\delta$c 127.6), C-9 ($\delta$c 146.1) (Figure 2). Therefore, **1** was elucidated as shown, and named buxruguline A.

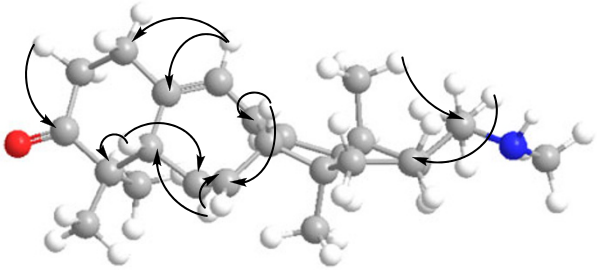

**Figure 2.** Key HMBC correlations for compound **1**.

Buxruguline B (**2**) was obtained as colorless needles. The HRESIMS exhibited a quasi-molecular ion peak at *m/z* 370.3105 ($[M + H]^+$, calc. 370.3109), indicating the molecular formula $C_{25}H_{39}NO$. The $^1H$ NMR spectrum featured three singlets for the three tertiary methyl groups at $\delta_H$ 0.85, 0.90, and 1.08, and the characteristic cyclopropyl methylene protons appeared as two doublets at $\delta_H$ − 0.04 and 0.50 (4.1). The $^1H$ and $^{13}C$ NMR spectrum of **2** displayed the presence of a terminal methylidene [$\delta_H$ 4.61, 4.85 (each, 1H, s); $\delta_H$ 153.3 (C), 101.5 ($CH_2$)]. All the data indicated that compound **2** was similar to buxpiine[7], and the distinct difference between them was that a oxygenated methine ($\delta_C \approx 72$) of C-16 in buxpiine was replaced by a methylene ($\delta_C$ 34.2) in **2**. This deduction was supported by HMBC correlations from H-21 ($\delta_H$ 1.08) to C-20 ($\delta_C$ 212.9), C-16 ($\delta_C$ 34.2) and from H-17 ($\delta_H$ 2.66) to C-20, C-16 and C-13 ($\delta_C$ 42.7). H-5 is invariably $\alpha$-oriented in this type alkaloid[18,19], the ROESY correlation of H-3 ($\delta_H$ 2.85) with H-5 ($\delta_H$ 2.12) indicating an $\alpha$-orientation of H-3 and $\beta$-orientation of the amino functionality. So, the structure of **2** was elucidated as shown in Figure 1.

Buxruguline C (**3**) had the molecular formula $C_{28}H_{48}N_2O_3$, as determined by HRESIMS analysis ($[M + H]^+$, *m/z* 461.3749). The $^1H$ NMR ($CDCl_3$) spectrum of **3** showed the presence of three methyl singlets at $\delta_H$ 1.08, 0.60, and 0.92 for the H-18, H-31, and H-32, a doublet methyl at $\delta_H$ 1.01 (6.0, 21-$CH_3$), two N-methyl singlets at $\delta_H$ 2.45 and 2.88, together with characteristic protons due to one hydroxymethylene ($\delta_H$ 2.93 and 3.25), and one oxygenated methine ($\delta_H$ 4.27). These spectral data were quite similar to those of dihydrocyclobaleabuxine[20], except for the resonance of acetyl group [$\delta_H$ 1.92; $\delta_C$ 172.2 (C) and 22.3 ($CH_3$)] attributable to the nitrogen at C-3. The relative configuration of **3** was elucidated by the ROESY experiment and comparision with other naturally occurring triterpenoid alkaloid possessing $\beta$-configuration of the amino group at C-3, H-5$\alpha$, and H-20$\beta$[18,19]. The ROESY correlations of H-5 ($\delta_H$ 1.84), and H-3 ($\delta_H$ 2.98) with H-30 ($\delta_H$ 2.93 and 3.25), and of H-20 ($\delta_H$ 2.97) with H-16 ($\delta_H$ 4.27) indicated that H-30 was in $\alpha$-orientation, while H-16 was in $\beta$-orientation, respectively. Thus, the structure of buxruguline C was established as **3**.

The moleculr formula of buxruguline D (**4**) was assigned as $C_{29}H_{48}N_2O_3$ on the basis of the NMR data (Table 1) and HRESIMS. Comparison of the spectroscopic data of **4** and **3** revealed similarities cycloartane-type triterpenoid skeleton. The notable difference was that a OH functionality at C-16 in **3** was replaced by acetoxy group in **4**, which confirmed by the downfielded H-16 ($\delta_H$ 4.11) proton signal and the HMBC correlation from H-16 to the O-acetyl carbonyl carbon at $\delta_C$ 169.3 (C). Moreover **4** has one less hydroxyl function at C-30 and one less methyl group on the nitrogen at C-3 than **3**. Consequently, compound **4** was elucidated as shown and has been accorded the trivial name buxruguline D.

Biologically, all compounds were tested for their cytotoxicity against the HL-60, SMMC-7721, A549, MCF-7, and SW480 cells lines (Table 2). Compounds **6**, **7** and **8** showed the better cytotoxic potential against A-549, and SW480 cell lines. Compounds **1**–**4** were noncytotoxic, with $IC_{50}$ values > 40 $\mu$mol for all tested cell lines.

## Experimental Section

**General Experimental Procedures.** Melting points were determined on a YU-HUA X-4 melting point apparatus. Optical rotations were obtained with a Horiba SEAP-300 polarimeter. Infrared spectra were recorded on a Shimadzu IR-450 instrument by using KBr pellets. NMR spectra were measured on a Bruker AV-400 and DRX-500 instrument (Bruker, Zűrich, Switzerland) with TMS as internal standard. HR-ESIMS data were recorded on a VG Auto Spec-3000 spectrometer. Silica gel (200–300 mesh, Qingdao Marine Chemical, Inc), amino silica gel (75–100 $\mu$m, Fuji Silysia Chemical LTD, Japan), C-18 (20–45 $\mu$m, Fuji Silysia Chemical, LTD, Japan), and Sephadex LH-20 (Pharmacia) were used for column chromatography.

**Plant Material.** *Buxus rugulosa* were collected at Lijiang (Yunnan), China, in February 2008. The sample was identified by Prof. Xi-Wen Li of the Kunming Institute of Botany, and a voucher specimen (KIB 20080210) has been deposited at the State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences.

**Extraction and Isolation.** The materials of *B. rugulosa* (75.0 kg) were extracted with 90% MeOH under reflux, the combined extracts were partitioned between EtOAc and 0.001 mol/L HCl (pH ≈ 3.0). The aqueous layer was alkalinized to pH 10.0 with 2 mol/L NaOH followed by exhaustively extraction with $CHCl_3$. The $CHCl_3$-soluble fraction (180 g) was chromatographed on a silica gel column, eluted with $CHCl_3$-MeOH (1:0-0:1), to give four fractions (FA–FD). FB (45 g) was chromatographed on silica gel using petroleum ether (PE)-EtOAc (8:1) as solvent and repeated Sephadex LH-20 eluted with MeOH to yield **7** (19 mg). After column chromatography on C-18 gel column chromatography by aqueous MeOH (60%-90%), amino silica gel column with PE-EtOAc (10:1) and $CHCl_3$-MeOH (50:1), further separated by Sephadex LH-20 eluted with MeOH, FC (14 g) to afford **1** (11 mg), **4** (4 mg), **5** (6 mg), **7**(28 mg), **8** (42 mg). FD (12 g) was chromatographed on silica gel using $CHCl_3$-MeOH (10:1, 5:1) as gradi-

ent, and was further repeatedly separated on amino silica gel column chromatography, eluted with $CHCl_3$-MeOH (20:1, 10:1), to give **2** (6 mg), **3** (12 mg), **6** (76 mg).

**Buxruguline A (1):** white powder; mp 188–190°C; $[\alpha]_D^{24}$ + 15.6 (*c* 1.04, $CHCl_3$); UV (MeOH) $\lambda_{max}$ (log ε) 208 (3.78), 244 (2.21) nm; IR (KBr) $\nu_{max}$: 1734 $cm^{-1}$; $^1H$, $^{13}C$ NMR data see Table 1; EIMS *m/z*: 367, HRESIMS *m/z*: 368.2944 $[M + H]^+$ (calcd for $C_{25}H_{38}NO$ $[M + H]^+$, 368.2953).

**Buxruguline B (2):** colorless needle; mp 223–224 °C; $[\alpha]_D^{24}$ + 18.4 (*c* 1.21, $CHCl_3$); UV (MeOH) $\lambda_{max}$ (log ε) 205 (3.59) nm; IR (KBr) $\nu_{max}$: 1735 $cm^{-1}$; $^1H$, $^{13}C$ NMR data see Table 1; ESIMS *m/z*: 370 $[M + H]^+$; HRESIMS *m/z* 370.3105 (calcd for $C_{25}H_{40}NO$ $[M + H]^+$, 370.3109).

**Table 1. $^1H$ and $^{13}C$ NMR Spectral Data of Buxrugulines A–D (1–4).**

| | **1**[a] | | **2**[b] | | **3**[c] | | **4**[a] | |
|---|---|---|---|---|---|---|---|---|
| position | $\delta_C$, type | $\delta_H$ | $\delta_C$, type | $\delta_H$ | $\delta_C$, type | $\delta_H$ | $\delta_C$, type | $\delta_H$ |
| 1a | 29.7, $CH_2$ | 2.04, overlap | 31.0, $CH_2$ | 1.82, overlap | 32.4, $CH_2$ | 1.56, overlap | 31.6, $CH_2$ | 1.65, overlap |
| 1b | | 1.90, overlap | | 1.46, overlap | | 1.17, overlap | | 1.47, overlap |
| 2a | 46.6, $CH_2$ | 2.34, m | 25.7, $CH_2$ | 1.73, overlap | 27.0, $CH_2$ | 1.55, overlap | 25.6, $CH_2$ | 1.33, overlap |
| 2b | | 1.84, m | | 1.65, m | | | | 1.08, overlap |
| 3 | 206.8, C | | 63.6, CH | 2.85, m | 51.3, CH | 2.98, overlap | 65.5, CH | 3.75, m |
| 4 | 44.3, C | | 153.3, C | | 44.0, C | | 42.2, C | |
| 5 | 41.1, CH | 2.73, d (3.5) | 41.9, CH | 2.12, m | 40.2, CH | 1.84, s | 50.5, CH | 2.16, s |
| 6a | 128.4, CH | 5.62, m | 21.2, $CH_2$ | 1.80, overlap | 20.5, $CH_2$ | 1.62, overlap | 20.9, $CH_2$ | 1.50, overlap |
| 6b | | | | 1.18, overlap | | | | 0.76, overlap |
| 7a | 127.6, CH | 5.42, m | 26.2, $CH_2$ | 1.58, overlap | 25.4, $CH_2$ | 1.22, overlap | 28.4, $CH_2$ | 1.75, m |
| 7b | | | | 1.14, overlap | | 1.12, overlap | | 1.49, overlap |
| 8 | 41.5, CH | 1.91, overlap | 42.4, CH | 2.00, m | 47.5, CH | 1.35, overlap | 47.3, CH | 1.44, overlap |
| 9 | 146.1, C | | 23.3, C | | 19.0, C | | 19.6, C | |
| 10 | 146.3, C | | 32.0, C | | 25.5, C | | 25.8, C | |
| 11a | 127.7, CH | 5.69, m | 21.4, $CH_2$ | 1.50, overlap | 25.8, $CH_2$ | 2.04, m | 26.1, $CH_2$ | 1.33, overlap |
| 11b | | | | 1.16, overlap | | 1.04, overlap | | 1.08, overlap |
| 12a | 30.2, $CH_2$ | 1.60, overlap | 33.3, $CH_2$ | 1.72, overlap | 31.7, $CH_2$ | 1.62, overlap | 32.2, $CH_2$ | 1.22, overlap |
| 12b | | 1.50, m | | 1.55, overlap | | 1.44, overlap | | |
| 13 | 39.5, C | | 42.7, C | | 45.6, C | | 44.9, C | |
| 14 | 41.5, C | | 46.0, C | | 47.7, C | | 48.0, C | |
| 15a | 24.8, $CH_2$ | 2.04, overlap | 22.4, $CH_2$ | 1.52, overlap | 45.5, $CH_2$ | 1.82, overlap | 45.0, $CH_2$ | 1.38, overlap |
| 15b | | 1.58, overlap | | 1.18, overlap | | 1.34, overlap | | |
| 16a | 26.0, $CH_2$ | 1.32, overlap | 34.2, $CH_2$ | 2.12, m | 75.8, CH | 4.27, m | 78.5, CH | 4.11, m |
| 16b | | | | 1.17, overlap | | | | |
| 17 | 47.5, CH | 1.88, overlap | 46.8, CH | 2.66, m | 56.1, CH | 1.93, m | 57.0, CH | 1.88, m |
| 18 | 19.7, $CH_3$ | 0.80, s | 18.3, $CH_3$ | 0.85, s | 18.9, $CH_3$ | 1.08, s | 19.0, $CH_3$ | 0.97, s |
| 19 | 129.7, CH | 6.51, s | 22.0, $CH_2$ | 0.50, d (4.1) | 30.0, $CH_2$ | 0.51, d (4.1) | 30.2, $CH_2$ | 0.53, d (4.1) |
| | | | | − 0.04, d (4.1) | | 0.32, d (4.1) | | 0.35, d (4.1) |
| 20 | 68.1, CH | 2.09, m | 212.9, C | | 64.1, CH | 2.97, overlap | 62.7, CH | 2.71, m |
| 21 | 15.3, $CH_3$ | 0.76, d (6.5) | 17.8, $CH_3$ | 1.08, s | 10.3, $CH_3$ | 1.01, d (6.0) | 9.8, $CH_3$ | 0.91, d (6.5) |
| 30a | 20.5, $CH_3$ | 1.26 (s) | 101.5, $CH_2$ | 4.85, s | 63.7, $CH_2$ | 3.25, m | 11.4, $CH_3$ | 0.76, s |
| 30b | | | | 4.61, s | | 2.93, m | | |
| 31 | 22.6, $CH_3$ | 1.17, s | | | 10.9, $CH_3$ | 0.60, s | 19.0, $CH_3$ | 0.97, s |
| 32 | 25.8, $CH_3$ | 1.05, s | 11.5, $CH_3$ | 0.90, s | 20.3, $CH_3$ | 0.92, s | 21.1, $CH_3$ | 1.15, s |
| 3-$NCH_3$ | | | 34.5, $CH_3$ | 2.50, s | 35.3, $CH_3$ | 2.45, s | | 2.30, s |
| 20-$NCH_3$ | 35.3, $CH_3$ | 2.47, s | | | 30.5, $CH_3$ | 2.88, s | | |
| N*C*OCH$_3$ | | | | | 172.2, C | | 171.4, C | |
| NCO*CH*$_3$ | | | | | 22.3, $CH_3$ | 1.92, s | 21.0, $CH_3$ | 2.09, s |
| O*C*OCH$_3$ | | | | | | | 169.3, C | |
| OCO*CH*$_3$ | | | | | | | 23.6, $CH_3$ | 1.96, s |

[a] $^1H$ and $^{13}C$ NMR spectra were acquired at 500 ($CDCl_3$) and 125 MHz ($CDCl_3$), respectively. [b] $^1H$ and $^{13}C$ NMR spectra were acquired at 400 ($CDCl_3$) and 100 MHz ($CDCl_3$), respectively. [c] $^1H$ and $^{13}C$ NMR spectra were acquired at 400 [$CDCl_3$:MeOD (1:1)] and 100 MHz [$CDCl_3$:MeOD (1:1)], respectively.

**Table 2. Cytotoxicity data of compounds 1–8 with $IC_{50}$ values ($\mu$M).**

| No. | HL-60 | SMMC-7721 | A-549 | MCF-7 | SW480 |
|---|---|---|---|---|---|
| **1** | > 40 | > 40 | > 40 | > 40 | > 40 |
| **2** | > 40 | > 40 | > 40 | > 40 | > 40 |
| **3** | > 40 | > 40 | > 40 | > 40 | > 40 |
| **4** | > 40 | > 40 | > 40 | > 40 | > 40 |
| **5** | 27.18 | > 40 | > 40 | 27.03 | > 40 |
| **6** | 15.23 | 28.99 | 19.39 | 14.39 | 14.69 |
| **7** | 17.32 | > 40 | 19.70 | > 40 | 14.25 |
| **8** | 21.35 | > 40 | 23.52 | > 40 | 17.18 |
| cisplatin (MW300) | 1.00 | 17.05 | 26.75 | 14.97 | 16.88 |

**Buxruguline C (3):** colorless needle; $[\alpha]_D^{24}$ + 8.8 (*c* 0.89, $CHCl_3$); UV (MeOH) $\lambda_{max}$ (log ε ) 205 (3.53) nm; IR (KBr) $\nu_{max}$: 1698 $cm^{-1}$; $^1H$, $^{13}C$ NMR data see Table 1; ESIMS *m/z* 461 $[M + H]^+$; HRESIMS *m/z* 461.3749 (calcd for $C_{28}H_{49}N_2O_3$ $[M + H]^+$, 461.3743).

**Buxruguline D (4):** white powder; $[\alpha]_D^{24}$ + 17.5 (*c* 0.72, $CHCl_3$); UV (MeOH) $\lambda_{max}$ (log ε ) 203 (3.67) nm; IR (KBr) $\nu_{max}$: 1696 $cm^{-1}$; $^1H$, $^{13}C$ NMR data see Table 1; EIMS *m/z* 472 $[M + H]^+$; HRESIMS *m/z* 473.7114 (calcd for $C_{29}H_{49}N_2O_3$ $[M + H]^+$, 473.7109).

**Cell Culture and Cytotoxicity Assay.** A panel of human tumor cell lines was used: promyelocytic leukemia HL-60, hepatocellular carcinoma SMMC-7721, alveolar basal epithelial carcinoma A549, breast adenocarcinoma MCF-7, and colon cancer SW480. The cells lines were obtained from the Shanghai cell bank of China. All the cells were cultured in RPMI-1640 or DMEM medium (Hyclone, USA), supplemented with 10% fetal bovine serum (Hyclone, USA) at 37°C in a humidified atmosphere with 5% $CO_2$.

Cell viability was assessed by conducting colorimetric measurements of the amount of insoluble formazan formed in the living cells with the MTT (MTT, sigma, USA) method described before[21], and using cisplatin (DDP, sigma, USA) as control. Cell growth inhibition curve was graphed and the $IC_{50}$ value of each compound was calculated by the Reed and Muench method[22].

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